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Use of Hydrogen Peroxide Vapor for Deactivation of *Mycobacterium tuberculosis* in a Biological Safety Cabinet and a Room[▽]

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***Mycobacterium tuberculosis* is an important human pathogen that is routinely cultured in clinical and research laboratories. *M. tuberculosis* can contaminate surfaces and is highly resistant to disinfection. We investigated whether hydrogen peroxide vapor (HPV) is effective for the deactivation of *M. tuberculosis* on experimentally contaminated surfaces in a biological safety cabinet (BSC) and a room. Biological indicators (BIs) consisting of an ~3-$\log_{10}$ inoculum of *M. tuberculosis* on stainless steel discs and a 6-$\log_{10}$ inoculum of *Geobacillus stearothermophilus* were exposed to HPV in BSC time course experiments and at 10 locations during room experiments. In three separate BSC experiments, *M. tuberculosis* BIs were transferred to growth media at 15-min intervals during a 180-min HPV exposure period. No *M. tuberculosis* BIs grew following 30 min of HPV exposure. In three separate room experiments, *M. tuberculosis* and *G. stearothermophilus* BIs were exposed to HPV for 90, 120, and 150 min, respectively. BIs for both microorganisms were deactivated in all 10 locations following 90 min of HPV exposure. HPV provides an alternative to traditional decontamination methods, such as formaldehyde fumigation, for laboratories and other areas contaminated with *M. tuberculosis*.**

Mycobacterium tuberculosis is an intracellular, pleomorphic, acid-fast bacillus that causes tuberculosis (TB) (14). Approximately one-third of the world's population is infected with *M. tuberculosis*, which causes two million deaths per annum (8, 29). TB is closely associated with human immunodeficiency virus coinfection, and TB is the most common cause of death due to AIDS (8, 29). The significance of TB has increased further due to the emergence of multidrug-resistant *M. tuberculosis* (9, 35).

Mycobacteria are notoriously resistant to sterilization and disinfection procedures, largely due to an unusual hydrophobic cell wall (5, 33) and effective defense mechanisms against oxidative stress (30). Mycobacteria are more resistant to biocides than most other nonsporulating bacteria (16, 32, 34) and can survive for extended periods on environmental surfaces (2, 24, 39). These factors present unique decontamination challenges to laboratories that culture *M. tuberculosis* for diagnostic or research purposes.

M. tuberculosis should be handled in a biosafety level (BSL) 3 laboratory (37), which is equivalent to containment level 3 in the United Kingdom (1). BSL 3 and containment level 3 laboratories must be sealable for vapor-phase decontamination. Formaldehyde fumigation has traditionally been used to decontaminate laboratories and biological safety cabinets (BSCs) used to handle *M. tuberculosis* (28), but there are significant safety and efficacy concerns associated with formaldehyde fumigation (6, 7, 11).

Hydrogen peroxide vapor (HPV) has been used increasingly for the biodecontamination of BSCs and rooms in health care, pharmaceutical, and other applications (19, 20, 22, 23). The HPV system described in this study has key advantages over traditional vapor-phase methods, such as formaldehyde fumigation, including uniform distribution via an automated dispersal system, a relatively rapid cycle time, a good safety profile, and external cycle control. Several studies have demonstrated the biological efficacy of HPV against bacterial endospores (20, 31), vegetative bacteria (13), viruses (17), and other microorganisms, including prions (12). A recent study demonstrated the tuberculocidal efficacy of HPV (21). We investigated further the tuberculocidal efficacy of HPV by determining whether a different, condensation-based HPV system could deactivate *M. tuberculosis* on experimentally contaminated surfaces in a BSL 3 laboratory, and we defined the point of kill using time course studies in a BSC.

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MATERIALS AND METHODS

Preparation and enumeration of biological indicators. *M. tuberculosis* biological indicators (BIs) were prepared to investigate the efficacy of HPV. *M. tuberculosis* H37Rv (ATCC 27294) was subcultured in mycobacterial growth indicator tubes (MGIT) (Becton Dickinson, Sparks, MD), and 3 days after signaling, 1 ml was washed with saline and colony counts performed on Middlebrook 7H10 agar. Ten microliters of an ~10^5 CFU/ml suspension was applied to the center of 10-mm, presterilized, stainless steel discs (Apex Laboratories, Inc., Apex, NC) and air-dried. This inoculum was selected because our laboratory procedure for the handling of a *M. tuberculosis* spill dictates that the bulk spill be cleaned using liquid disinfectant prior to vapor-phase fumigation; we therefore felt that a 10^3-CFU inoculum was representative of the likely concentration of contamination postcleaning. Control- and HPV-exposed *M. tuberculosis* BI disks were transferred into mycobacterial growth indicator tubes, inverted gently several times to mix, and cultured in a BACTEC MGIT 960 system (Becton Dickinson) for 44 days to determine if any viable organisms remained. Positive tubes were confirmed using *M. tuberculosis*-specific nucleic acid hybridization probes (AccuProbe MYCOBACTERIUM TUBERCULOSIS Complex culture identification test; Gen-Probe, San Diego, CA). *M. tuberculosis* BIs were transported

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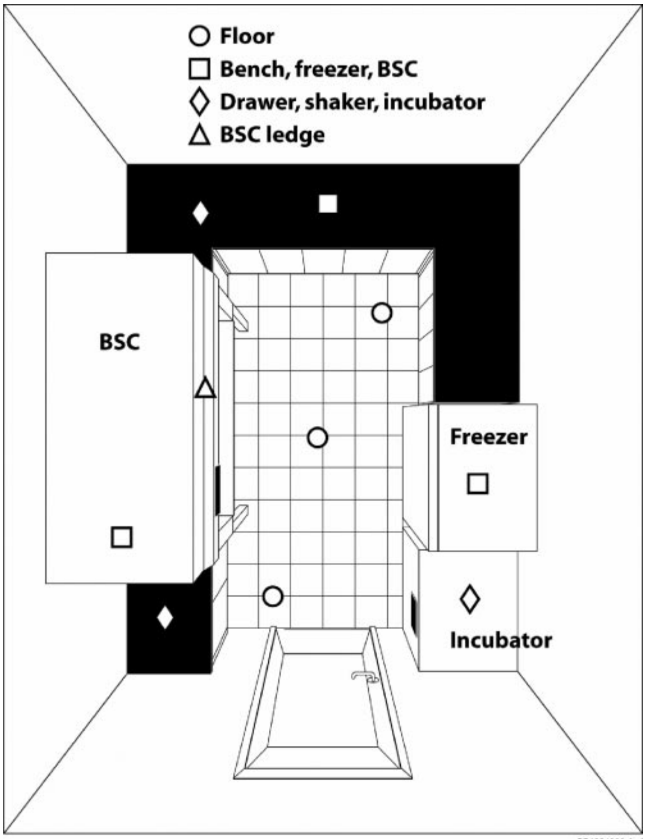

FIG. 1. Schematic of the BSL-3 laboratory with BI placement locations.

in petri dishes, and the lids were removed when the BIs were placed in the BSC or room.

Geobacillus stearothermophilus BIs with a population of $>1.0 \times 10^6$ on stainless steel discs sealed in individual Tyvek pouches (Apex Laboratories, Inc., Apex, NC) were used to confirm the efficacy of the HPV room decontamination cycles. Control- and HPV-exposed *G. stearothermophilus* BIs were cultured in 20 ml Trypticase soy broth (TSB) at 60°C and examined for turbidity over 7 days.

BSC time course experiments. A ducted class II BSC (type B; Nuaire, Plymouth, MN) in a BSL 3 laboratory was used to conduct the time course experiments. The duct was closed using a manual damper system, and the cabinet was retrofitted with a modified front panel, which included a glove port to facilitate manipulation of the materials inside the BSC. An HPV sensor (Analytical Technology, Inc., Collegeville, PA) was inserted through a port in the modified front panel. A Clarus S HPV suite (BIOQUELL, Inc., Philadelphia, PA) was used to decontaminate the BSC. The suite consists of a vaporizer to produce and distribute HPV generated from 30% (wt/wt) liquid hydrogen peroxide (Fisher Scientific, Pittsburgh, PA) and internal and external catalytic units to effect pressure control on the BSC and catalyze the decomposition of HPV to oxygen and water vapor. The HPV decontamination cycle consists of the HPV injection phase, in which the liquid H_2O_2 is vaporized and distributed throughout the BSC; the HPV dwell phase, in which no further HPV is injected but active HPV distribution continues within the BSC by means of a fan on the vaporizer; and the aeration phase, in which HPV is catalytically converted to oxygen and water vapor. The BSC duct was opened during the aeration phase to accelerate the aeration process. The Clarus S HPV suite is configured to operate at negative pressure to prevent HPV leakage, and the exterior of the cabinet was monitored for leakage using a handheld HPV sensor (PortaSens II; Analytical Technology, Inc., Collegeville, PA).

Three replicate cycles with a 180-min HPV exposure period were conducted in the BSC. *M. tuberculosis* BIs were placed inside the work chamber of the BSC and were transferred into MGIT tubes (Becton Dickinson) inside the BSC at 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, and 180 min. A tube was opened inside the BSC at 50 min for 10 s to approximate the time required to inoculate a BI into a MGIT tube. This tube was then removed from the BSC at the end of the cycle and used to culture an unexposed BI as an exposure control to confirm that a significant amount of HPV was not absorbed into the culture media during the transfer of the BI. A BI was exposed to room air outside the BSC during the HPV exposure period and transferred to culture media at 180 min as a dry control to ensure that desiccation alone did not deactivate the BIs. An uninoculated, unopened negative control broth was incubated to confirm the sterility of the media.

BSL 3 laboratory room experiments. The room trials were conducted in a 1,320-ft^3 (37-m^3) BSL 3 laboratory. The room was decontaminated using the Room Bio-Decontamination Service (BIOQUELL, Inc., Philadelphia, PA), which utilizes four portable modules: the Clarus R HPV generator, the Clarus R2 aeration unit, an instrumentation module, and a control computer. The Clarus R HPV generator is placed within the room and produces HPV from 30% (wt/wt) liquid H_2O_2 (Fisher Chemicals, Pittsburgh, PA). The resulting HPV is delivered via a dual-axis vapor distribution system that ensures high kinetic energy and even distribution throughout the room. The HPV concentration, temperature, and relative humidity within the room are measured by the instrumentation module and monitored by a control computer situated outside the room. When HPV exposure is complete, Clarus R2 aeration units inside the room catalytically convert the HPV into oxygen and water vapor.

The heating, ventilation and air-conditioning (HVAC) system in the room was disabled to prevent unwanted dispersion or dilution of the HPV during the exposure period. The HVAC extraction system was reinstated during the aeration phase to accelerate the process. The perimeter of the laboratory was monitored for leakage using the PortaSens handheld HPV sensor.

Three cycles were conducted in the BSL 3 laboratory, with HPV exposure periods of 90, 120, and 150 min. In each cycle, *M. tuberculosis* and *G. stearothermophilus* were placed at the following 10 locations in the laboratory: three bench-tops, floor in the middle and corner of the laboratory, work chamber (front panel open and BSC fans off), freezer lid, inside a shaker with the lid ajar by approximately 4 in., ledge of the BSC, and inside a drawer with the lid ajar by approximately 2 in. (Fig. 1). The BIs were removed from the room at the end of the aeration phase, transferred to their respective media, and cultured.

RESULTS

BSC time course experiments. The three replicate BSC decontamination experiments had an HPV exposure period of 180 min, consisting of a 50-min injection phase followed by a

TABLE 1. *M. tuberculosis* biological indicators exposed to HPV in a BSC and removed to broth at timed intervals

Cycle	Signal			Signal at HPV exposure time (min) of:												
	Exposure control[a]	Dry control[b]	Negative control[c]	0	15	30	45	60	75	90	105	120	135	150	165	180
1	+	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−
2	+	+	−	+	−	−	−[d]	−	−	−	−	−	−	−	−	−
3	+	+	−	+	+	−[d]	−	−	−	−	−	−[d]	−[d]	−[d]	−	−

[a] Exposure control broth was opened inside the BSC at 50 min for 10 s, removed from the BSC at the end of the cycle, and used to culture an unexposed BI.
[b] The dry control BI was exposed to room air outside the BSC during the HPV exposure period and transferred to culture media at 180 min.
[c] Negative control broth was incubated to confirm the sterility of the medium.
[d] Tube signaled MGIT positive. All subcultures and AFB stains were negative. Duplicate nucleic acid hybridization probes were negative.

TABLE 2. Technical cycle data from the three room experiments

HPV exposure time (min)	Temp at start of cycle (°C)	Temp at end of injection phase (°C)	RH at start of cycle (%)	RH at end of injection phase (%)	H_2O_2 delivered (g)	Peak HPV concn (ppm)	Total cycle time (inc. aeration[a]) (min)
90	25.5	28.6	29.5	55.1	916	>1,000	210
120	29.7	32.4	26.6	50.9	1,193	>1,000	270
150	27.4	31.4	33.0	56.1	1,479	>1,000	330

[a] inc., including; aeration was ended at 2 ppm. The powerful HVAC system rapidly removed the residual HPV from the air.

130-min dwell phase. The total cycle time including aeration was approximately 5 h. The peak HPV concentration in the cabinet was in excess of 2,000 ppm, which was the upper limit of the HPV sensor used. Visible condensation was noted in the BSC during the injection phase, which persisted into the dwell phase but was dissipated by the end of the aeration phase. No HPV leakage was detected outside the BSC.

All time zero BIs and exposure and dry controls signaled on the MGIT machine and were TB probe positive. The negative control did not signal on the MGIT machine (Table 1). These results suggest that first, the *M. tuberculosis* BI system was functioning correctly; second, insignificant amounts of H_2O_2 were absorbed into the media during the transfer of BIs; and third, desiccation alone does not explain the deactivation of the *M. tuberculosis* BIs. In two of the three replicate experiments, *M. tuberculosis* BIs were positive at 15 min but negative at 30 min, suggesting that deactivation occurred between 15 and 30 min (Table 1). In the remaining experiment, deactivation occurred between 0 and 15 min (Table 1). Since the *M. tuberculosis* BIs had a population of 10^3 CFU as prepared, we can deduce that an approximate 3-$\log_{10}$ reduction of *M. tuberculosis* was achieved between 0 and 30 min in the BSC under the conditions tested. The HPV-exposed *M. tuberculosis* BIs signaled positive on the MGIT machine on 5 (17%) of the 29 negative BIs, which occurred on BIs from 30, 45, 120, 135, and 150 min of HPV exposure; 4/5 of these were from the third experiment, which was conducted on the same day immediately after the second experiment (Table 1). All five of these BIs were visually clear, failed to hybridize with the *M. tuberculosis*-specific nucleic acid probes (performed in duplicate), were acid-fast bacillus stain negative, and showed no growth following subculture on Middlebrook 7H10/selective 7H11 agar biplates and incubation at 37°C for 60 days, suggesting false positives. False-positive results are noted as possible in the manufacturer's product insert and have been reported at low frequency in other studies using the MGIT system (40).

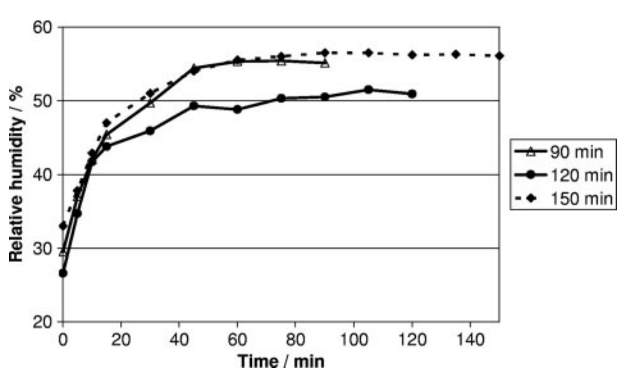

FIG. 2. Increasing relative humidity during the HPV injection phase of the room experiments.

BSL 3 laboratory room experiments. The mean temperature and relative humidity (RH) in the room rose from 27.5°C and 29.7%, respectively, at the beginning of the cycle to 30.8°C and 54.1%, respectively, at the end of the injection phase (Table 2). The RH increased rapidly at the beginning of the HPV injection phase, and the rate of change tended towards zero during the injection phase in each of the three cycles (Fig. 2). The RH returned to approximately 30% at the end of the aeration cycle due to the dehumidifying effect of the Clarus R2 aeration units, but the temperature remained elevated due to the motors operating the Room Bio-Decontamination Service equipment in the room. The 90-, 120-, and 150-min HPV exposure cycles delivered 916, 1,193, and 1,479 g of H_2O_2 and had total cycle times of 3.5, 4.5, and 5.5 h, respectively (Table 2). The peak HPV concentration in each cycle was in excess of 1,000 ppm (Table 2), which was the limit of the HPV sensor used.

The *M. tuberculosis* time-zero BI and the drying control were both MGIT and probe positive and the negative control was MGIT negative in all three room experiments, suggesting that the *M. tuberculosis* BI system was functioning correctly (Table 3). The *G. stearothermophilus* positive controls grew and the negative control did not grow in all three room experiments (Table 3). A drying control was not conducted for *G. stearothermophi-*

TABLE 3. *M. tuberculosis* and *G. stearothermophilus* biological indicators exposed to HPV in a 1,320-ft^3 room

BI	Signal at HPV exposure time (min) of:		
	90	120	150
Dry control[a]	+	+	+
Negative control[b]	–	–	–
Bench-top 1	–	–	–[c]
Bench-top 2	–	–	–[c]
Bench-top 3	–	–	–
Floor, middle	–	–	–
Floor, corner	–	–	–
BSC work chamber	–	–	–
Freezer lid	–	–	–[c]
Shaker (ajar)	–	–	–
Ledge of BSC	–	–	–
Drawer (ajar)	–	–	–

[a] The dry control BI was exposed to room air outside the BSC during the HPV exposure period and transferred to culture media at 180 min (not done for *G. stearothermophilus* BIs).

[b] Negative control broth was incubated to confirm the sterility of the media.

[c] Tube signaled MGIT positive. All subcultures were negative. Duplicate nucleic acid hybridization probes were negative.

lus, because the BIs have a certified shelf life from the manufacturer.

M. tuberculosis and *G. stearothermophilus* BIs were deactivated in the 10 locations tested in all 3 room experiments (Table 3). This suggests that the distribution of the HPV in the room was adequate and that the shortest exposure period (90 min) was sufficient to deactivate the BIs. Three (10%) of 30 negative exposed *M. tuberculosis* BIs from the 150-min HPV exposure cycle signaled MGIT positive but were visually clear and negative by subculture, acid-fast-bacillus staining, and probe hybridization, suggesting that they were false positives (Table 3). *M. tuberculosis* BIs had a population of $\sim 10^3$ CFU, and *G. stearothermophilus* BIs had a population of $>1.0 \times 10^6$ CFU; hence we can deduce that an approximate 3-$\log_{10}$ reduction of *M. tuberculosis* and a $>$6-$\log_{10}$ reduction of *G. stearothermophilus* were achieved by less than or equal to 90 min of HPV exposure in a static BSL 3 laboratory.

DISCUSSION

The intrinsic resistance of mycobacteria to physical stress is high, and there are several reasons to suspect that the resistance of mycobacteria to HPV will be especially high. Mycobacteria produce the *katG* catalase-peroxidase that catalyzes the breakdown of H_2O_2 and have other defenses against oxidative stress to facilitate their intracellular survival (30). Although the exact mechanism of action of HPV remains to be fully elucidated and probably varies with microorganisms, H_2O_2 generates oxidative stress by producing reactive oxygen species, such as hydroxyl radicals, that attack multiple molecular targets, including nucleic acids, enzymes, cell wall proteins, and lipids (18, 26, 27). Mycobacterial oxidative defense mechanisms would interfere with the bactericidal action of HPV. Also, the HPV system used in this study is configured to form condensation that is often invisible to the naked eye ("microcondensation"), and the hydrophobic cell wall of mycobacteria will resist the formation of such H_2O_2 condensation on the cell.

Therefore, although the HPV system is rapidly sporicidal (20, 31), we used extended cycles for both the BSC and room trials to account for the anticipated high resistance of *M. tuberculosis*. A 3-$\log_{10}$ reduction of *M. tuberculosis* was achieved in less than 30 min in the BSC experiments and in less than 90 min in the room experiments. Another study using a different HPV system also found that a 5-$\log_{10}$ inoculum of *M. tuberculosis* was deactivated by a 90-min HPV exposure period (21). Other in vitro studies have demonstrated that *M. tuberculosis* can be killed by H_2O_2 (10) or H_2O_2-based disinfectants (34). Therefore, it seems that the oxidative defense mechanisms and robust structure of *M. tuberculosis* are overcome by H_2O_2.

Full deactivation of *M. tuberculosis* and *G. stearothermophilus* BIs was achieved at the 10 locations in all three room experiments. BIs for each organism were placed inside a drawer that was opened by approximately 2 in. Similarly, BIs were placed inside a platform shaker with the lid propped open by approximately 4 in. All of these BIs were deactivated, suggesting that the dual-axis vapor distribution system of the Clarus R instrument distributes the HPV effectively.

The initial rapid increase and subsequent plateau of the RH in the room experiments (Fig. 2) is consistent with the formation of "microcondensation" on surfaces in the room. Saturation and subsequent condensation are observed at 100% RH in a water vapor/air system. The introduction of H_2O_2 and its associated hydrogen bonding affects this dynamic and reduces the dew point so that saturation and subsequent condensation can occur at RH levels considerably lower than 100%, depending on the starting conditions (38). The condensation-based HPV process relies upon direct contact between condensed H_2O_2 and the microorganism. Theoretical analysis of decontamination cycles shows that this condensation is critical for rapid and repeatable decontamination (38). Similarly, high relative humidity ($>$50%) been found to be critical for the successful formaldehyde fumigation (36). While calculated saturation conditions were achieved in the room experiments and the RH profile was consistent with the formation of condensation, visible condensation was seen only in the BSC, suggesting that "microcondensation," invisible to the naked eye, was formed during the room experiments.

There was no surface damage in the BSC or room despite three long cycles. A computer that was left running in the room was fully functional following the three room experiments. We did not experience any other safety issues, which alleviates the concerns raised by Kahnert and colleagues, who proposed that "condensation (from the gas to a liquid phase) of hydrogen peroxide can be damaging to surfaces and presents safety risks in particular in room decontamination" (21). Furthermore, the condensing HPV system used in this study has been deployed in many other settings without damage to sensitive electronics, surface damage, or safety risks associated with "microcondensation" (13, 19, 20).

We developed a BI system to test the HPV sensitivity of *M. tuberculosis* dried on experimentally contaminated stainless steel surfaces. The BI system was designed to represent contamination that may occur in laboratories during routine culture of *M. tuberculosis* or following spillage of *M. tuberculosis* in liquid culture and subsequent cleanup of the spillage using liquid disinfectants as per local procedures. We chose to use a BI-broth system rather than enumeration of each HPV-exposed *M. tuberculosis* BI for several reasons. First, recovery from dried bacterial culture is unlikely to be 100%, so the count of *M. tuberculosis* is most likely an underestimate. Second, broth culture is more sensitive because, theoretically, a single viable cell of *M. tuberculosis*, which may not be detected during an enumeration procedure, would multiply and signal on the MGIT machine. Third, the BI-broth system reduced the necessity for open plate culture of *M. tuberculosis*, which was attractive for health and safety reasons. Overall, 14% of the *M. tuberculosis* BIs were false positives, which is higher than the previously reported rate of 0.7% (40) and is higher than the false-positive rate seen in our clinical cultures. All three of the room false positives occurred after the longest exposure period (Table 3), and most of the BSC false positives occurred in discs transferred to the MGIT broths later in the cycle (see Tables 1 and 3). Furthermore, four of the five BSC false positives were from the third cycle, which was run on the same day immediately after the second cycle, so the elevated temperature and relative humidity may have resulted in more condensation than with the first two experiments. Although the reason for these false positives is not completely understood, hydrogen peroxide is a weak acid in solution, so residual hydrogen peroxide transferred on the discs may have caused a pH shift affecting the MGIT fluorescent-indicator system. However, the exposure control was positive, demonstrating that HPV absorbed during the transfer of the discs did not affect the ability of *M. tuberculosis* to grow in the MGIT tubes.

The implementation of HPV decontamination in a busy clinical laboratory has several practical considerations. Although HPV has a safety profile superior to those of other vapor-phase high-level disinfectants, specifically with regard to toxicity and diffusibility, certain controls are required to ensure the health and safety of all personnel and continued efficacy of the process. Controls include perimeter monitoring using handheld HPV sensors to detect leakage, external control of the HPV equipment so that immediate aeration is possible if necessary, and the use of *G. stearothermophilus* BIs to verify the efficacy of each room cycle. All personnel must vacate the laboratory room being decontaminated with HPV, but adjacent laboratories can continue to operate as usual. BSCs are held at negative pressure during HPV decontamination so that others can continue to work in the same laboratory. The long room cycles that we used resulted in the room becoming uncomfortably hot and humid. Although the heat and humidity were returned to comfortable levels within a few hours by reinstatement of the HVAC system, the development cycles used in our study were longer than typical HPV cycles. It is hoped that future cycle optimization will demonstrate that shorter cycles, which will result in smaller increases in heat and humidity, are equally efficacious.

Our study has several limitations. First, we used only one strain of *M. tuberculosis* and did not investigate other species of mycobacteria; studies with liquid disinfectants have found that *Mycobacterium avium* complex is more resistant to liquid disinfectants than *M. tuberculosis* (16). The strain that we used is not multidrug resistant, whereas clinical isolates of *M. tuberculosis* are increasingly multidrug resistant. Multidrug-resistant *M. tuberculosis* often possesses an impaired catalase-peroxidase system because *katG* mutation is involved in isoniazid resistance (3), so it is reasonable to suspect that these organisms will be less resilient to HPV. Further, there are a number of publications indicating that drug resistance in *M. tuberculosis* confers a fitness cost compared with susceptible strains (4, 15, 25). We would therefore hypothesize that drug-resistant *M. tuberculosis* should be equally susceptible or perhaps even more susceptible to HPV than drug-susceptible *M. tuberculosis*. Our preliminary research provides a good foundation for research into *M. tuberculosis* with impaired catalase-peroxidase systems or other mechanisms of drug resistance. Second, we did not enumerate every BI and assumed that the *M. tuberculosis* BIs used for each experiment had a comparable inoculum based on preliminary studies (data not shown). Third, shorter cycles in both the BSC and room experiments would have provided more-defined information regarding the sensitivity of *M. tuberculosis* to HPV. Future work should investigate intra- and interspecies variation of HPV sensitivities among mycobacteria, especially multidrug-resistant strains, and define the deactivation time more accurately.

Our study has shown that a condensing HPV decontamination system is effective for the deactivation of *M. tuberculosis* and *G. stearothermophilus* dried onto stainless steel surfaces in a BSC and a BSL 3 laboratory. HPV provides an alternative to traditional methods, such as formaldehyde fumigation, for the decontamination of laboratories used to handle *M. tuberculosis.* HPV could also be applied in the health care setting for the terminal decontamination of isolation facilities used to care for patients with tuberculosis.

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